Maximizing Total RNA Yield from TRIzol® Reagent Protocol: A Feasibility Study

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ABSTRACT

This work aimed to optimize RNA isolation from samples of limited size using TRIzol® reagent. Investigated were the effects of certain variables, especially the amount of TRIzol® reagent used in homogenization of samples and exposure to freezing conditions, on total RNA isolation from vertebrate tissue. Initially, it was possible to optimize the amount of TRIzol® used for the initial homogenization of amputated zebrafish caudal fin tissue. Samples were then prepared in three groups; RNA was isolated immediately following homogenization (0 hour) and after 1 hour and 24 hours of being snap- frozen in a -80°C freezer. Lastly, since extraction of RNA in the aqueous phase involves elution with 24:1 chloroform/ isoamyl alcohol after the initial homogenization step of the protocol, also examined was how the volume of chloroform/ isoamyl alcohol impacted yield. The experimental techniques were then applied to isolating RNA from regenerated zebrafish tissue 24 hours post- amputation of the caudal fin for a study investigating gene regulation in regenerated tissue. Functional studies included cDNA synthesis and sequential RT-PCR to evaluate quality mRNA extraction by employing these optimized methods. After additional validation, the techniques were then applied to extraction of total RNA from a single zebrafish embryo at 4- cell, high, and 24 hpf stages of development. It was observed that this optimization process yields quality RNA (RIN ~ 10.0), and there exists significance in the amount of reagents applied to a sample versus the amount of RNA obtained from the extractions.

INTRODUCTION

In molecular biology, isolating quality RNA is a critical first step in any analytical follow up processing technique such as Reverse Transcription PCR (RT- PCR) or Quantitative/ Real Time PCR (qPCR) as well as Northern Blot analysis of transcripts. Obtaining sufficient starting material for cDNA synthesis is heavily dependent on proper precautions when handling RNA, but even more important is ensuring that the chosen protocol optimizes yield as well as quality. Often it is the less abundant RNA species that are sought after for analysis. There are multiple established products available through commercial vendors that offer reliability in nucleic acid yield. New England Biolabs offers a magnetic isolation kit which exploits the poly(A) tail in mRNA and is most effective when used on cells and tissue [1] which improves greater amounts of primary transcripts in the starting sample. Qiagen Technologies' RNeasy Mini Kit utilizes silica- membrane embedded spin columns for binding capacities up to 100 µg of single strand material. Each offers a unique mechanism for isolating quality RNA.

The wholesome and accurate isolation of mRNA is important in studying microRNA (miRNA) and their

regulatory functions in morphogenesis [2]. Furthermore, careful screening of these small RNA fragments in small amounts of sample tissue could lead to breakthroughs in protocols for tissue engineering as well as therapies for cancer malignancies [3]. Most functioning cancer pathways have been proven to be regulated by relatively small traces of mRNA sequences [4- 7], it is therefore crucial to recover the majority of the RNA in such samples for further processing.

The zebrafish is an effective animal model for studying regenerative tissue processes through various molecular signaling events, and could potentially be of significant importance to biomedical engineers for studying stem cell involvement in such occurrences. The zebrafish has the ability to regrow caudal fin tissue while sustaining tissue regrowth for a defined timeline post- amputation, and slight alterations in cell signaling factors could potentially offer explanations for the inability of these events to occur rapidly and freely in humans. Gene expression from early embryonic stages throughout adult development of the zebrafish must be studied in a quantifiable manner should therapeutics be derived from this animal model for human oriented applications. In one study, a process was developed for maximal RNA isolation from a single zebrafish embryo at various stages of development whereas most methods called for 10 embryos at the minimum for sufficient RNA yield [8]. Establishment of inexpensive yet reliable isolation methods for maximal RNA yield is significant, and adds to the toolbox of the modern researcher.

TRIzol® reagent from Invitrogen is an economical yet effective phenol based medium that is used by many investigators primarily involved in molecular biology and the biotechnology disciplines. After homogenization of tissue or cells, chloroform is used to extract RNA from the homogenate in an aqueous layer and all remaining protein complexes are collected in a lower phenol- red formed organic layer. The isolation process using this reagent can be successfully completed in less than one hour, and assuming sterile technique can be used to extract RNA in pure form (A260/280 wavelength ratio greater than or equal to 1.9). This chemical reagent can be used to thoroughly isolate RNA from bodily fluids, tissues, and cells, and has been used to extract RNA from plasma and the nucleic acid recovered was then utilized in qPCR reactions [9]. Even from cervical samples, isolated RNA for use in the analysis of HPV oncogenes which are normally costly to detect *in vivo* was then carried to the next step after purity analysis for cDNA synthesis [10]. One study used TRIzol® reagent as a co- extraction method for isolating RNA from formalin-fixed paraffin-embedded specimens that had been stored for extended amounts of time, and showed significant quality starting material for PCR to further investigate the archived specimens in subsequent studies [11].

The goal of this work was to optimize RNA extraction from small portions of regenerated caudal fins to improve analysis of cell signals during regeneration. RNA extraction from single cell embryos was also optimized.

MATERIALS AND METHODS

1. Developing an Optimal Protocol for Extraction of RNA from Zebrafish Caudal Fin Tissue.

1A. Optimizing Initial TRIzol® Volume for a Defined Tissue Sample.

A standard amount of zebrafish caudal fin was amputated and homogenized in varying amounts of TRIzol® in order to isolate total RNA. In the preliminary isolation experiments, areas of amputated fin tissue from zebrafish were measured using image J software (NIH Bethesda, Maryland) and then amputated accordingly. Volumes of TRIzol® reagent in sterile centrifuge tubes at 50, 100, 150, 200, 250 µL volumes were chilled on ice prior to homogenization of the tissue. Tissue was homogenized using a mechanical pestle (Fisher Scientific, item#12-141-368), and extracted using manufacturer's recommended protocol. Concentration and purity (A260/ 280 ratio) readings were read using a NanoDrop Lite Spectrophotometer from Thermo Scientific.

1B. Testing the effect of Freezing Samples prior to RNA extraction on yield.

Fin tissue was amputated as stated above and two sets (50, 100, 150, 200, 250 µL) were frozen in a -80°C

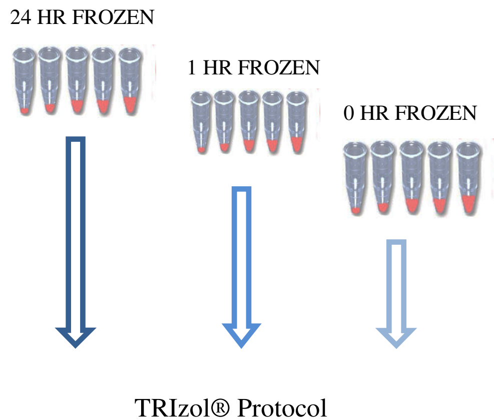


Figure 1. Flow Diagram of freezing experiments.

freezer for 1 and 24 hour periods. RNA was isolated using manufacturer's recommended protocol (FIG 1).

1C. Testing the Effect of Chloroform volume on yield.

The original instructions found in the manufacturer's protocol calls for the addition of 0.2 mL of chloroform to each sample for every 1.0 mL of TRIzol® reagent used in homogenization. For experimental tests, 0.4 and 1 mL of 24:1 chloroform/ isoamyl alcohol was used for every 1 mL of TRIzol® reagent in the phase extraction step (20 and 50 µL respectively).

FINAL OPTIMAL PROCEDURE FOR RNA ISOLATION FROM FIN TISSUE

Prepare a sterile 1.5 mL centrifuge tube prior to beginning the protocol with 50 µL of TRIzol® reagent stored on ice. TRIzol® reagent contains phenol (toxic and corrosive) and guanidine isothiocyanate (an irritant), and may be a health hazard if not handled properly (reference Invitrogen Life Technologies website for more information on safety measures).

1| Adult zebrafish (2+ years old) can be anesthetized using diluted Tricaine before tissue amputation proceeds being careful not to overdose the fish, and monitoring for gill movement throughout the procedure.
! CAUTION: Use of zebrafish will require IACUC approval at a researcher's institution.
2| Amputate an area of the caudal fin that you wish to isolate RNA from and immediately place into the 50 µL volume of TRIzol® reagent prepared earlier.
! CAUTION: This should be performed on a sterile surface and with a sterile cutting utensil.
3| Using a mortar and pestle, completely homogenize the tissue ● **TIMING 1- 2 min**
▲CRITICAL STEP: Ensure tissue is submersed in the TRIzol® reagent for complete disassociation.
■ PAUSE POINT: homogenate can be frozen for 24 hours at -80°C at this point if not able to continue.
4| Add 20 µL of 24:1 chloroform/ isoamyl alcohol to the homogenate and vigorously shake for 15 seconds.
5| Centrifuge for 15 min at 12, 000 x g in 4°C.
6| Extract the aqueous layer that results after centrifuging to a fresh centrifuge tube.
▲CRITICAL STEP: Be sure to extract the aqueous layer carefully; any lower organic phase contamination greatly decreases final product purity. Overall yield requires efficient aqueous layer removal during this step.
7| Add 25 µL of isopropanol to the extracted aqueous layer, and mix by inverting 2- 3 times.
8| Centrifuge for 10 min at 12, 000 x g in 4°C.
9| Decant the supernatant. Given the total volume, gentle tapping on a solid surface may be needed to aspirate.
10| Add 50 µL of 75 % cold ethanol (in DEPC treated water) to the centrifuge tube, and mix by inverting 2- 3 times.
11| Centrifuge for 5 min at 7,500 x g in 4°C.
12| Decant the supernatant. Given the total volume, gentle tapping on a solid surface may be needed to aspirate.
13| Air dry the RNA ● **TIMING 5- 10 min**
! CAUTION: Incomplete drying during this step with result in purity readings above 2.0 (A260/ 280).
14| Resuspend the RNA in 20 µL of UltraPure™ nucleotide- free distilled water.
▲CRITICAL STEP: Place the RNA suspension on ice for approximately 5 min to completely dissolve the RNA.

Obtain a concentration and purity reading on a Nanodrop spectrophotometer

2. Validating RNA Quality Through Reverse Transcription (RT)-PCR.

After validation of the experimental procedure for maximizing RNA yield with specific reagent parameters, the next approach aimed to test the ability for such materials to be used in Reverse Transcriptase (RT)-PCR. Zebrafish caudal fins were amputated, and regular regrowth of fin tissue proceeded until 24 hours post- amputation (FIG 5). At this point, regenerated tissue was collected from the fin and RNA isolation was performed using the optimized protocol described in the previous section. Samples were reverse transcribed with Oligo(dT)$_{20}$ primer to yield cDNA using the SuperScript™ III First- Strand Synthesis kit (Invitrogen Carlsbad, CA). β-actin expression was assayed using 4.5 µg of RNA and Forward primer 5'– GGAGAAGATCTGGCA TCACACCTTCTAC–3'and Reverse Primer: 5'– TG GTCTCGTGGATACCGCAGATTCCAT–3' used as a loading control. The PCR reactions were analyzed by gel electrophoresis and imaged under UV trans-illumination with AlphaEase® FC software from Alpha Innotech.

3. Developing an Optimal Protocol for Extraction of RNA from a Single Zebrafish Embryo.

Embryos were collected at the 4- cell, high (3 hours post fertilization (hpf), and 24 hpf stages of development. The single embryo was homogenized in a tube of 50ul of TRIzol® as described in the protocol developed in section 1 of methods. The RNA samples from the 4- cell and 24 hpf embryos were assayed for RNA quality, by obtaining an RNA integrity number (RIN) from Boston Children's Hospital, CORE Facility, utilizing an Agilent Bioanalyzer (Agilent Technologies, INC.).

FINAL OPTIMAL PROCEDURE FOR RNA ISOLATION FROM A SINGLE EMBRYO

For this protocol, a brief washing step is required before further RNA isolation can precede.

1| Transfer one embryo to a sterile 1.5 mL centrifuge tube with as little water as possible.

2| Add 1 mL of ddH$_2$O (distilled/ deionized) to the tube, and place on an electric inverter ● TIMING **2 min**

3| Blank your spectrophotometer using the ddH$_2$O used for washing, and test the washed embryo solution following the inversion step.

4| Repeat step **2** and **3** until the RNA concentration in the solution approaches the blanking level before proceeding to homogenization, allowing for adequate cleaning of the embryo.

5| Gently remove as much water as possible, making sure to spot the embryo in the tube during the removal.

6| Add 50 µL of TRIzol® reagent to the embryo tube.

7| Using a mortar and pestle, completely homogenize the embryo ● TIMING **5- 10 sec**

▲CRITICAL STEP: Ensure embryo is submersed in the TRIzol® reagent for complete disassociation.

Continue with the modified protocol as explained in earlier RNA isolation methods (see previous PROCEDURE).

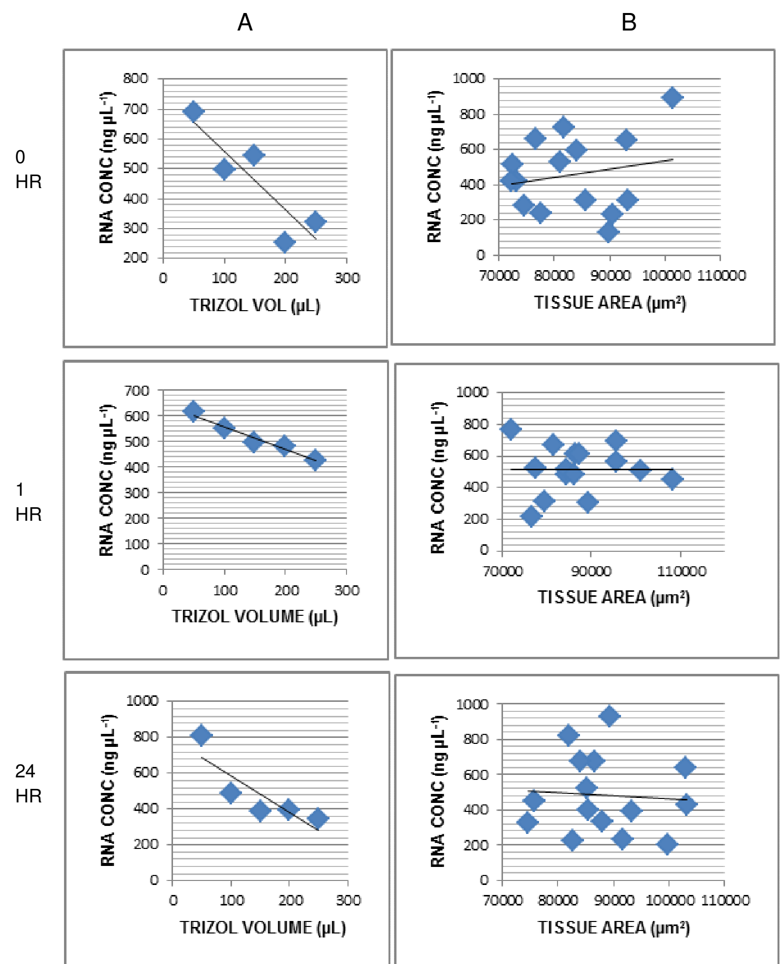


Figure 2. Effects on RNA concentration as a function of area of tissue amputated, volume of TRIzol® reagent used during homogenization, and snap freezing time in the -80°C freezer. RNA concentration as a function of volume of TRIzol® reagent showed a strong negative correlation across all snap freezing times accounting for the majority of the variation ($r^2 > 0.70$; $p < 0.001$) with the strongest correlation in 1 HR frozen conditions ($r^2 \sim 0.97$; $p < 0.001$). Area appeared to not affect RNA concentration significantly in each condition ($r^2 < 0.04$; $p > 0.05$).

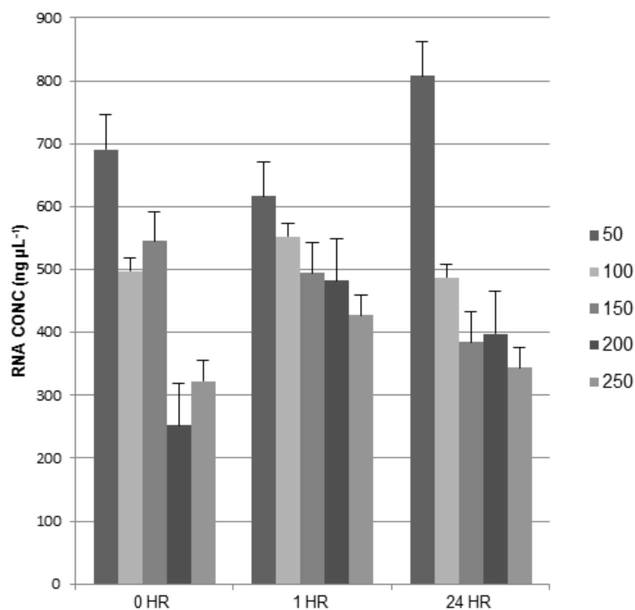


Figure 3. In varying the amount of TRIzol® reagent used for homogenization, a significant trend was observed in RNA concentration by ANOVA analysis ($F > F_{CRITICAL}$; $p < 0.001$). There appeared to be no significant difference in RNA yield due to varying snap freezing conditions. As the volume of TRIzol® reagent used in homogenization increased, RNA concentration generally decreased. Results represented as the average and standard deviation.

RESULTS AND DISCUSSION

The overall goal of these experiments was to evaluate variables present in the original TRIzol® protocol that could be optimized and potentially maximize quality RNA yield if applied and if the potential risk of losing valuable genetic material due to miniscule amounts of collected sample is high. The following introduces some key factors in extracting the maximum amount of RNA from the tissue samples based on the previously discussed data.

Optimizing Trizol volume and assaying the effect of freezing on RNA isolated from Zebrafish Caudal Fin Tissue

A standard amount of fin tissue was used to determine the optimal amount of TRIzol® to use for homogenization in order to extract the highest amount of RNA. This range was established to investigate the effect on final RNA concentrations versus dilution of samples in solution. The effect of freezing samples after homogenization in TRIzol® was also examined to determine if there was any consequence to not performing the whole extraction in freshly homogenized samples. As expected we saw no significant degradation in RNA or decrease in overall purity as a result of snap freezing experiments [12]. During these steps, it was important to amputate tissues as swiftly and efficiently as possible, since environmental heat denaturing of the RNA can alter future quantitative analysis [13]. It is important to note that complete homogenization of the fish tissue is critical during these steps, and on average took 1-2 minutes to completely dissociate tissues. Data revealed that deviations in area readings during amputation resulted in no significant correlation to the amount of RNA isolated during the protocol (FIG 2B) whereas at all three freezing conditions there appeared to be a strong negative correltation to RNA concentration versus increased amount of TRIzol® reagent used in homogenization (FIG 2A). Overall, homogenizing the tissue in 50 µL of TRIzol® reagent resulted in the highest concentrations in total RNA content across all freezing conditions (FIG 3). Two-way ANOVA analysis proved no significant difference in RNA concentration as a result of the amount of time in freezing or as a result in error in

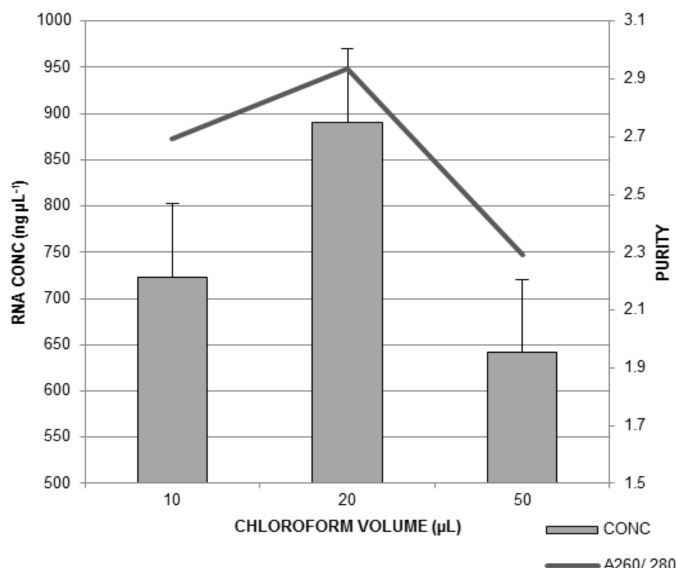


Figure 4. Using three different volumes of 24:1 chloroform/ isoamyl alcohol (10, 20, and 50 µL) in the extraction step of the manufacturer's protocol, a significant total RNA peak in concentration was observed in the 20 µL volume used (0.4 mL ratio to every 1 mL TRIzol® reagent). Optimal average purity was also assigned to the 20 µL volume and concentration. However, all purity readings were recorded to be above 2.0. Results represented as the average and standard deviation.

tissue area ($F < F_{CRITICAL}$), while confirming a significant difference in RNA concentration as affected by the volume of TRIzol® reagent used in homogenization ($F > F_{CRITICAL}$; $p < 0.001$). All purity readings were recorded to be 1.9 and above by standard spectrophotometry.

Testing the Effect of Chloroform extraction volume on yield and quantity.

The next measure of this study focused on further optimization on RNA yield in varying the amount of chloroform/ isoamyl alcohol used in the phase separation segment of the protocol. The original instructions found in the manufacturer's protocol calls for the addition of 0.2 mL of chloroform to each sample for every 1.0 mL of TRIzol® reagent used in homogenization. For experimental tests, 0.4 and 1 mL of 24:1 chloroform/ isoamyl alcohol was used for every 1 mL of TRIzol® reagent in the phase extraction step (20 and 50 µL respectively). Results from this variable in the extraction step show that by aspirating with 20 µL of the 24:1 chloroform/ isoamyl alcohol, RNA yield was increased by 23± 11% (n=5) when compared with the original control volume of the chloroform/ isoamyl alcohol suggested in the protocol instructions. However in using the 50 µL chloroform/ isoamyl alcohol extraction volume, RNA yield was seen to be at the lowest concentration (approximately 11% less than the 10 µL extraction) and also the lowest average purity. Maximum purity was recorded with the 20 µL extraction versus the other two extraction volumes (FIG 4). The 20 µL extraction volume was seen to have a significantly greater RNA concentration yield as compared to the other two volumes used in extraction method ($F > F_{CRITICAL}$; $p < 0.001$). From these data, it is possible to conclude that RNA is most efficiently extracted from tissues using TRIzol® reagent at low volumes depending on how minuscule of a sample intended to extract RNA from. In doubling the amount of 24:1 chloroform/ isoamyl compared to the amount recommended, a significant increase in RNA yield was obtained in the procedure. As an observation in extracting RNA with the 1:1 ratio TRIzol® reagent: chloroform/ isoamyl alcohol, it was difficult to see a clear phase separation as seen normally in lower volumes (10 and 20 µL). The lower phenol- red layer appeared dilute, but the top extracted layer was collected as thoroughly as possible. This could have accounted for the lower purity reading for this volume used in extraction (FIG 4).

However, it is important to note that snap- freezing RNA sample in TRIzol® reagent does not appear to jeopardize RNA concentration and purity as indicated by our results (FIG 3). Therefore, should a discontinuity in the isolation protocol be necessary, it is possible to delay the process for as long as a 24 hour period after homogenizing and RNA yield along with purity will still be at desired quality levels as indicated by the data outlined in the previous section.

Validating the quality of isolated RNA through Reverse Transcriptase PCR.

After validation of the experimental procedure for maximizing RNA yield with specific reagent parameters, the next approach aimed to test the ability for such materials to be used in RT- PCR. RT-PCR assays the amount of cDNA reverse transcribed from the isolated RNA. This is a common method to

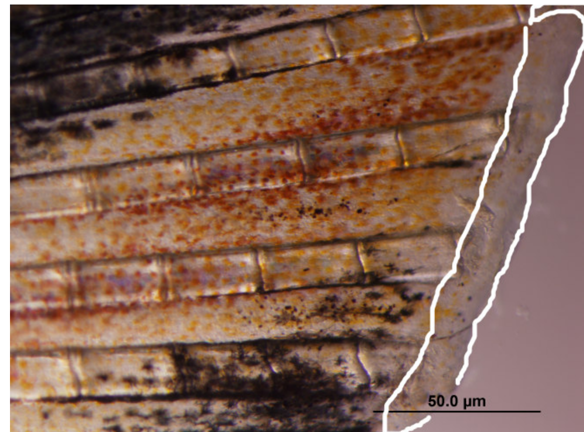


Figure 5. Zebrafish caudal fins were amputated, and observed 24 hours post amputation for regenerative tissue outgrowth (indicated by white line).

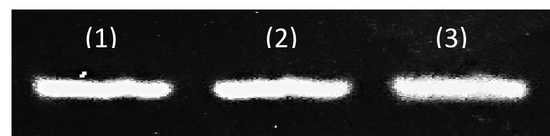
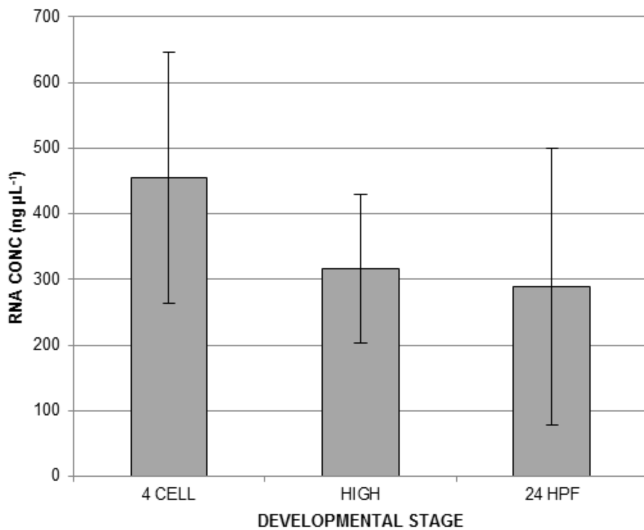


Figure 6. RNA from regenerative zebrafish tissue was isolated and reverse transcribed to yield cDNA. End- point PCR was then performed to evaluate qualitatively the usability of the isolated material. Replicates indicated as (1), (2) and (3) as shown.

assay gene expression in tissues and is dependent on quality RNA isolation and lack of RNA degradation. At this point, regenerated tissue was collected from the fin (FIG 5) and RNA isolation was performed using optimized protocol 1 described in the previous section. Through this protocol, yields greater than 500 ng μL^{-1} were obtained. After cDNA synthesis, we successfully amplified a 197 bp actin gene fragment (FIG 6). This result indicated a quality mRNA yield from the process that could be utilized for consistent future analytical works such as end-point PCR.

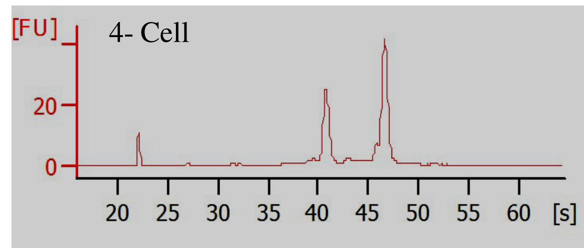
RNA Isolation from a Single Zebrafish Embryo

The study then focused on optimizing RNA yield from a single zebrafish embryo using the optimized protocol which further validated the process. Results proved that relatively large amounts of RNA can be isolated from a single embryo during early stages of development (FIG 7). The maximum amount of total RNA isolated across all stages was obtained from the 24 hpf embryo (13.5 µg; n = 5), while the highest total average was obtained consistently from the 4-cell embryo (9.1 µg; n = 5). Results indicated high levels of quality RNA (FIG 8). Homogenization for an extended period is not needed here given the fragile nature of the early stage embryo. Otherwise, all other adjustments used throughout the optimized protocol were carried out regularly for this experimental portion.



EMBRYO STAGE	TOTAL RNA (µg)	A260/ 280
4- CELL	9.1 ± 3.8	2.16
HIGH	6.3 ± 2.3	1.92
24 HPF	5.8 ± 4.2	1.92

Figure 7. RNA isolation from a single embryo at three stages of development. Using the optimized protocol, over 5.0 µg of total RNA on average was isolated across the three stages with relative purities within optimal range (A260/ 280 > 1.7). Results represented by the average and standard deviation.



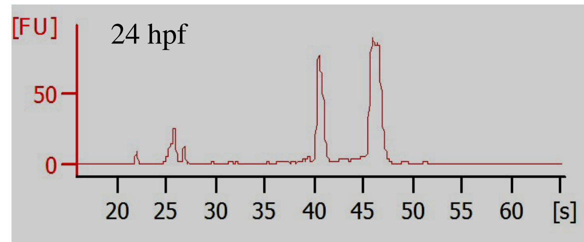


Figure 8. Pico- chip chromatogram confirmation of RNA Integrity. The RNA integrity numbers (RIN) for 4- cell and 24 hpf were 9.50 and 9.90 respectively (RIN range 1- 10; 10 is highest integrity RNA).

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